THESIS

THERMAL INACTIVATION OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 IN HEAT TREATED, RECYCLED USED COOKING OIL

Submitted by

Rachel Lynn Murphy

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2016

Master's Committee:

Advisor: Robert J. Delmore

Dale R. Woerner
Jennifer N. Martin
Ann Hess
Keith E. Belk

ABSTRACT

THERMAL INACTIVATION OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 IN HEAT TREATED, RECYCLED USED COOKING OIL

Rendered oils and fats are commonly used in pet food products. Given the implementation of the Food Safety Modernization Act (FSMA), which enforces a zero tolerance policy for *Salmonella* in all pet foods, the microbiological safety of these products has become an area of focus for the industry. Objectives of this study were to (i) understand lethality of a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat treated, recycled used cooking oil, (ii) estimate D- and z-values for a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat treated, recycled used cooking oil, and (iii) establish time/temperature processing steps targeting a selective set of strains of *Salmonella* and *E. coli* O157:H7 for use as preventive controls in a product safety plan. Objectives were addressed by inoculating previously rendered, recycled used cooking oil, with a five-strain mixture of *Salmonella* or *E. coli* O157:H7 at a level of approximately 8.0 log CFU/ml. Inoculated samples were exposed to heat at 62, 71, or 82°C using a water bath fitted with a circulator. At predetermined time intervals, individual samples were removed from water bath and immediately chilled in an ice bath. Samples were diluted in 0.1% buffered peptone water supplemented with 1% emulsifier (Tween 80). Samples were plated on selective agars (xylose lysine deoxycholate [XLD, *Salmonella*]; sorbitol MacConkey [SMAC, *E. coli* O157:H7]) and a non-selective agar (tryptic soy agar supplemented with sodium pyruvate at a level of 1%). A traditionally used aqueous inoculum would not be adequately distributed throughout an oil matrix and could potentially interfere with heat transfer during heat treatment.

Thus, pathogen cells were resuspended in warmed (37°C), sterile vegetable oil (soybean oil). No difference in inoculation level was observed when an aqueous solution and sterile vegetable oil were compared as inoculum matrices. Volumetric measurement of oil samples proved inconsistent and, thus, the mass was used to measure samples for experimentation. Addition of Tween 80 aided in homogenization of sample and diluent during surface plating and eliminated phase separation and errors in dilution (i.e. no ten-fold differences across dilutions). Additionally, a larger width tube (50 ml Falcon Tube) was used for the first dilution blank and vortexing was standardized to 30 s—these components proved to be critical for microbial analysis of high-fat liquids or semi-solids. Six replicates were performed for each pathogen and temperature combination. Data from surviving populations was utilized to generate thermal death curves. Segmented regression was performed using the Proc NLIN function of SAS (v 9.4) and z-values were calculated using linear regression (SAS v 9.4). Non-linear death curves were observed for all pathogen and temperature combinations. This response to heat treatment indicated not only pathogen survival, but also possible heat tolerance among some strains. As expected, D-values were lower as temperature increased and, for all pathogens, ranged from 0.03 to 0.04 min at 82°C, 0.14 to 0.27 min at 71°C, and 0.77 to 1.49 min at 62°C. Z-values ranged from 14.14°C to 27.78°C for all pathogens. Recommended time/temperature processing steps established from these data act as preventive controls in heat treatment of recycled used cooking oil. Processing times range from 7.46 min to 0.20 min.

ACKNOWLEDGMENTS

I would like to thank all my fellow graduate students for helping me through graduate school. All the long days, trips to the plant, taste panels, cutouts, and more were not only opportunities for us to grow as meat scientists but also for us to really get to know each other. I truly value all the time I spent with each and every one of you and for all the life lessons I gained. You all helped me grow as a person and I would not trade my experiences for anything.

Thank you to my advisor, Dr. Robert Delmore for always believing in me and pushing me to be the best version of myself. Thank you to Dr. Dale Woerner for inspiring me to strive for excellence and for being a strong mentor over the last two years. Thank you to Dr. Jennifer Martin for always being there as a friend and professor and for always knowing something about everything. Thank you to Dr. Ifigenia Geornaras for training me and guiding me through the world of food safety. My project would not have happened without your guidance and mentorship. I will truly miss having you as my teacher.

Thank you to my family—mom, Dan, Jenna, Laura, Baba, and Poppie. I made it to this point in my life because of you all. Thank you to Eric Romero for dealing with my crazy life over the last two years. Your support carried me through.

Thank you to Dr. Keith Belk for leading our group and being on my committee. I would like to thank Dr. Ann Hess for being my statistics teacher and committee member.

DEDICATION

This thesis is dedicated to my Poppie, Dr. Richard Mitchell. You instilled in me the importance of education and a love for learning. Without your continual love and support, I would not be the woman I am today. It is impossible to put into words all the lessons you taught me. You will always be my Poppie. I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Literature Review

1.1 History of the Rendering Industry

Rendering is the process of recycling and repurposing animal tissues and cooking fats and oils into a array of value-added products (Bisplinghoff, 2006). Every year in the United States and Canada, fifty-six billion pounds of inedible (unsuitable for human consumption) material generated by the livestock, meat and poultry, food processing, supermarket, and restaurant industries are recycled and repurposed by renderers (Bisplinghoff, 2006; National Renderers Association, 2015). Without the recycling service that renderers provide, landfills would quickly be overwhelmed with waste (Meeker and Hamilton, 2006; Meeker and Meisinger, 2015). It is estimated that without the rendering process, all available landfill space would be taken over within four years (National Renderers Association, 2015). A large fraction of rendered finished products are returned to the livestock and companion animal industries in the form of high-energy fats and high-quality protein ingredients to supplement the diets of swine, cattle, poultry, cats, and dogs (Meeker and Meisinger, 2015). Products of rendering are used in some cosmetics, biofuel, pharmaceuticals, fertilizers, soaps, and other products (McGlashan, 2006). The rendering process is a safe and economical method of destroying harmful bacterial populations while recovering useful, marketable commodities (Bisplinghoff, 2006).

Long before the advent of the meat industry, inedible animal by-products were used by humans (Franco, 2002; Bisplinghoff, 2006; Ockerman and Basu, 2015). In prehistoric times, inedible by-products served as clothing, fuel, fertilizer, and housing. In more modern generations, increased food animal production generated increased amounts of by-products.

These by-products became a cumbersome waste and disposing of them, especially in dense population areas, became an issue. The need for rendering was not only driven by the need to dispose of by-products, but also by the societal demand for tallow—tallow was the primary source for both soap and candles until the early twentieth century (Bisplinghoff, 2006; Ockerman and Basu, 2015). Generally, the protein fraction of rendering was treated as waste or sometimes was used as fertilizer (Bisplinghoff, 2006). In 1901, research was published by Professor C.S. Plumb at Purdue University that showed that swine had dramatically increased growth rates when their corn diet was supplemented with animal protein extracted during the rendering process (Ockerman and Basu, 2015). At the time, swine were typically ready for market at nine months—with the addition of rendered animal protein to their diet, swine reached market weight at seven months of age (Bisplinghoff, 2006). Since this revolutionary discovery, research has continued to show benefits of feeding rendered by-products, both the fat and protein portions, to livestock, poultry, companion animals, and fish (Meeker and Meisinger, 2015). With the establishment of large packing house centers such as Chicago and Omaha in the late 1800s, an increased demand for more sophisticated rendering equipment in order to process a large volume of raw material emerged (Bisplinghoff, 2006). Currently in the United States, there are over 180 rendering plants with 20 more operating in Canada (Meeker and Meisinger, 2015).

The rendering industry receives a majority of its raw materials from animal slaughter facilities (Jekanowski, 2011; Meeker and Meisinger, 2015). In the United States alone, 112 million hogs, 32 million cattle, and close to 10 billion chickens and turkeys are slaughtered each year (Meeker and Meisinger, 2015). Americans consider about 50% of cattle, 40% of hogs, and 30% of broiler chickens to be inedible—this includes bone, blood, fat, feathers, and some internal organs (Ockerman and Basu, 2014). It is expected that meat consumption will continue

to increase worldwide, with a majority of increased consumption seen in developing countries (FAO, 2009; Meeker and Meisinger, 2015). Since meat will continue to be a dietary component for humans around the world, rendering systems will continue to be a pinnacle in the sustainability of animal agriculture (Meeker and Meisinger, 2015).

1.2 Used Cooking Oil

Approximately 4.4 billion pounds of used cooking oil (UCO), a by-product of the restaurant, supermarket, and food processing industries, are sent to renderers annually (National Renderers Association, 2015a). There are two primary markets for UCO—biofuels and animal feeds, with biofuel currently taking the larger share. When used in livestock feed, it is commonly termed yellow grease if combined with low quality animal fats (Meeker and Hamilton, 2006; Pacific Alternative Energy Resource, n.d.). A small portion of UCO is also used in dried, extruded pet food (i.e., kibble) as a post-processing fat source (Aldrich, 2006; Plattner and Rokey, 2007; Nelles, 2008). Used cooking oil serves as not only an added fat and energy source, but also as a palatability enhancer, making the food more desirable to canines and felines (Aldrich, 2006; Nelles, 2008; Meeker and Meisinger, 2015). In the United States, approximately 2.7 billion pounds of rendered and recycled yellow grease and UCO are used annually in pet food production (Meeker and Hamilton, 2006).

In the rendering plant, UCO is filtered to remove any solids and then heated until it meets industry specifications (Jekanowski, 2011). Depending on the design of the facility, used cooking oil is either processed in separate, independent machinery, or is processed through the continuous cooker system that is used for animal products (250°F to 280°F; 121.1°C to 137.8°C). When UCO is processed through designated machinery (that not used for animal

products), UCO is heated in batches to a minimum of 180°F (82.2°C). Once the desired temperature is met, UCO is again filtered and stored until transport (via truck). Moisture, insoluble impurities, and unsaponifiables are the undesirable portions contained within UCO due to their negative effects on quality and nutritional content (BHT ReSources, 2016). Unsaponifiables are the lipid fraction of a fatty substance that cannot be transformed into soap; unsaponifiables consist of the non-hydrolysable components of the fatty acid substance and include sterols, hydrocarbons, fatty alcohols, and vitamins (Meeker and Hamilton, 2006; Laboratoires expanscience, n.d.). Moisture is detrimental to the quality of UCO since it speeds up the oxidation process and contributes to rust development on equipment (Meeker and Hamilton, 2006; BHT ReSources, 2016). During the heating process, the moisture fraction is removed and a typical composition of UCO includes 0.50% moisture (Dar Pro Solutions, n.d.). Insoluble impurities and unsaponifiables detract from the value of the UCO and can alter the nutritional quality of it (BHT ReSources, 2016). With filtration and heating, a large majority of the insoluble impurities and unsaponifiables are removed each with an average ending value of 0.50% (Dar Pro Solutions, n.d.). Possible insoluble impurities include small particles of fiber, bone, or even dirt (Meeker and Hamilton, 2006). Not only does the removal via filtering of insoluble impurities improve the quality of the end product, but it also reduces clogging of equipment and build-up of sludge in storage tanks (Meeker and Hamilton, 2006).

1.3 Companion Animal (Pet) Food

In companion animal diets, UCO serves as a source of concentrated energy, essential fatty acids, and a mechanism for absorption of fat-soluble vitamins while also improving texture, aroma, and flavor (Aldrich, 2006). Fat sources often add up to ten percent of the pet food formula (Aldrich, 2006). It is estimated that the pet food industry utilizes around 2.4 million tons

of rendered materials every year (Aldrich, 2006). In 2005, this accounted for roughly 25% of the total US production of rendered materials (Aldrich, 2006). This relationship indicates the strong dependency that the rendering and pet food industries have on each other.

Since UCO is commonly added after the extrusion process (but can be an inclusive ingredient), if there is bacterial contamination within the fats, there are no further intervention steps to control bacterial hazards. In 2012 during a FDA investigation of a pet food plant implicated in a *Salmonella* outbreak affecting both pets and humans, it was stated that incoming animal fat and oils could be the source of the contamination (Department of Health and Human Services, 2012). Companion animals, such as dogs and cats, can acquire pathogens via ingestion of contaminated feed, but may not present symptoms (Crump et al., 2002). Humans that come in contact with animals or their contaminated feed, in some cases via ingestion, can then become ill (Crump et al., 2002). Dogs and cats serve as a potential vector for salmonellosis via physical contact, environmental contamination, feces, bites or scratches, and other vector routes (Finley et al., 2006). Dogs and cats live in close proximity of their owners and families, increasing the level of contact and interaction (Sato et al., 2000; Finley et al., 2006). In 2002 it was reported that over 40% of dog-owners and over 50% of cat-owners share their bed with their pet—a dramatic increase of over 20% from 4 years earlier (Finley et al., 2006). The pet food industry is continually growing with over $60 billion in expected expenditures for 2015 (Carrion and Thompson, 2013; American Pet Products Association, 2016). According to the 2015-2016 American Pet Products Association Inc. National Pet Owners Survey, 65% of American households own at least one pet—a 10 percent increase from when the survey first began in the 1980s (American Pet Products Association, 2016). Although cases of human disease are rarely linked back to animal or pet feed, it is reasonable to assume that common strains of pathogens

cause disease via animal and pet food, but go undetected or uninvestigated (US General Accounting Office, 2000; Crump et al., 2002; Finley et al., 2006). With pet food, human and animal health converge. Besides the implications for human health, there is also concern with pet health. In 2007, the FDA was flooded with consumer complaints that cats and dogs were becoming ill and dying after consuming pet food (Maberry, 2016). The number of complaints received was more than double the typical yearly average prior to 2007 (Maberry, 2016). It was determined that pet food contaminated with melamine, an industrial chemical, was the causative agent (Maberry, 2016). The deaths of 8,000 cats and dogs indicated that unwholesome production of pet food can not only be harmful to animals but can also have large societal impacts (Maberry, 2016).

1.4 Food Safety Modernization Act (FSMA)

The Food Safety Modernization Act (FSMA) was signed into law by President Obama in January 2011 (Strauss, 2011). With FSMA, added funding and authority has increased the power of the FDA and the agency is now allowed to order recalls and have more oversight in plants (Strauss, 2011). The law was a move to try to assure consumers that their health and protection from dangerous foods is important (Strauss, 2011). A major component of FSMA requires that food and animal feed producers develop and implement preventive product safety plans. A prevention-based product safety plan requires a producer or processor to identify potential risks of contamination by physical, chemical, biological, and radiological hazards at all points throughout their process as well as identify methods of control (Strauss, 2011). The FDA described the change as saying that “For the first time, FDA will have a legislative mandate to require comprehensive, science-based preventive controls across the food supply” (U.S. Food and Drug Administration, 2016a). The final rule affecting the animal feed and rendering

industries was published in September 2015 and is titled “Current good manufacturing practice, hazard analysis, and risk-based preventive controls for food for animals” (U.S. Food and Drug Administration, 2016b). Very small businesses have up to 4 years to fully establish preventative controls while medium- to large-sized companies must be in full compliance by September 2017 (U.S. Food and Drug Administration, 2016b). The key requirements for producers or processors of animal feed is that they establish current good manufacturing practices (cGMPs; by September 2016), establish preventive controls, and complete a supply chain analysis (U.S. Food and Drug Administration, 2016b). It is required that facilities that further process by-products (the rendering industry) asses their processes to determine where hazards may exist that require preventative controls. Decisions must be based in science, and at the current time, there is limited scientific, research-based knowledge specifically for the rendering industry (U.S. Food and Drug Administration, 2016a). The main principles that are required with a written preventive food safety system, a mandatory element under FSMA, include identification of hazards, establishment risk-based preventive controls, monitoring of effectiveness, determination of appropriate corrective actions, perform verification, recordkeeping and documentation, a supply-chain program, and a requirement to reanalyze (FDA, 2015).

1.5 *Salmonella* spp.

The CDC estimated that there are approximately 1.2 million illnesses and 450 deaths caused by nontyphoidal *Salmonella* each year in the United States (Scallan et al., 2011; CDC, 2015b). On a global basis, it is estimated that 1.3 billion cases occur each year (Coburn et al., 2007). The organism was first discovered in 1885 by Theobald Smith and Daniel E. Salmon who were working for the newly formed Bureau of Animal Industry in the United States (Schultz, 2008). At the time, it was believed that this newly discovered species was the cause of cholera in

hogs (Schultz, 2008). The genus *Salmonella* is separated into two species: *S. enterica* and *S. bongori* with *S. enterica* being the greater risk to public health (FDA, 2012). *Salmonella enterica*, the significant species for human and animal health, is a non-sporeforming, gram-negative, facultative anaerobe (Coburn et al., 2007; FDA, 2012). The organism is further divided into subspecies which are further subdivided into serovars or serotypes (Coburn et al., 2007; FDA, 2012). *Salmonella enterica* is composed of six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI; Coburn, 2007; FDA, 2012). There are over 2,220 serovars within the genus of *Salmonella* that vary in their preferred hosts and ability to cause disease, despite their close genetic likeness (Baumler et al., 1998; Chan et al., 2003). More than 60% of all currently identified strains of *Salmonella* and 99% of the serovars that are responsible for disease in warm-blooded animals fall under subspecies I (*enterica*; Chan et al., 2003). Other subspecies are more common with cold-blooded animals and at times, systemic disease in humans (Chan et al., 2003). Serotypes or serovars are named following the Kaufmann-White typing scheme, published in 1934, which divides and differentiates serovars by their flagellar, carbohydrate, and lipopolysaccharide structures (Coburn et al., 2007; FDA, 2012; CDC 2015b).

The way the disease manifests is dependent upon not only host susceptibility but also upon the serovar (Humphrey, 2004; Coburn et al., 2007). The two types of illness caused by *S. enterica* are (1) nontyphoidal salmonellosis and (2) typhoid fever (Coburn et al., 2007; FDA, 2012). Typhoid fever is a serious illness with a high mortality rate, while nontyphoidal salmonellosis is generally self-limiting in healthy adults (FDA, 2012). Among immunocompromised populations, serovar *S.* Enteritidis (nontyphiodal salmonellosis) can have a mortality rate of 3.6% (FDA, 2012). Virulent *S. enterica* localize to the epithelium of the small

intestine, causing inflammation (Coburn et al., 2007; FDA, 2012). Onset of illness following exposure can range from 6 to 72 hours with the infective dose being as low as one cell; however, the infective dose can be greater than 50,000 cells in healthy adults (Coburn et al., 2007; FDA, 2012). Symptoms include nausea, vomiting, abdominal cramps, diarrhea, fever, and headache, all lasting 4 to 7 days (FDA, 2012; CDC, 2015b). Complications may include dehydration and electrolyte imbalances—only a major concern in immunocompromised populations (FDA, 2012). On rare occasions, approximately 2% of culture-proven cases, reactive arthritis (i.e. inflammation, urethritis, uveitis, or conjunctivitis) may follow 3 to 4 weeks after onset of symptoms (FDA, 2012; CDC, 2015b). *Salmonella* Dublin is sometimes associated with septicemia or bacteremia—the organism can possibly escape the gastrointestinal tract and spread the infection throughout the body and bloodstream (FDA, 2012). Pathogenic *Salmonella* uses an oral route of entry via contaminated food or water (FDA, 2012).

There are two serotypes of *Salmonella*, found only in humans, that cause typhoid fever—*S.* Typhi and *S.* Paratyphi A (FDA, 2012). If typhoid fever goes untreated, mortality rates can be as high as 10% (FDA, 2012). The infective dose is typically less than 1,000 cells with onset ranging from 1 to 3 weeks or even up to two months after exposure (FDA, 2012). Symptoms generally last 2 to 4 weeks and include a high fever, lethargy, gastrointestinal symptoms, and rose-colored rash (FDA, 2012). Route of entry and the manifestation of the illness are very similar to nontyphoidal salmonellosis (FDA, 2012). Septicemia, endocarditis, and/or chronic infection of the gallbladder may result from typhoid fever (FDA, 2012). In the United States, it is estimated that around 1,800 cases of typhoid fever occur annually from domestic sources while an additional 400 cases are acquired during international travel (FDA, 2012). The last foodborne

outbreak of typhoid fever in the United States occurred in 1999 from the tropical fruit mamey (FDA, 2012).

Virulence determines degree of pathogenicity; large regions of chromosomes termed "pathogenicity islands" define virulence characteristics of enteric bacteria like *Salmonella* (Ochman et al., 1996). *Salmonella* pathogenicity islands (SPIs) are critical for inducing inflammatory immune responses in infected hosts (Coburn et al., 2007). Two specific SPIs, 1 and 2, encode a molecular apparatus named type III secretion system (T3SS; Coburn et al., 2007). This T3SS is responsible for injecting bacterial proteins ("effectors") into host cells or the extracellular environment, thus allowing the organism to invade and infect the host (Chopra et al., 1999; Coburn et al., 2007). This is the known pathway for *S. enterica*—penetration and passage of the bacterial cells from the gut lumen of the intestines into the epithelium of the small intestine (FDA, 2012). The host reaction is to release phagocytic cells, resulting in inflammation (Chopra et al., 1999). The invasive cells are engulfed by the phagocytic cells and carried to the mesenteric lymph follicles where it multiplies thus providing a route of entry for infecting other organs (Chopra et al., 1999). The ability of *Salmonella* to induce intestinal fluid secretion is attributed, at least in part, to the production of an enterotoxin (Chopra et al., 1999). It is thought that the enterotoxin is produced within the enterocytes of the host (FDA, 2012). Interestingly, it has been shown that the *stn* gene, responsible for the production of enterotoxin, is present in *Salmonella* cells regardless of serovar indicating its involvement in the pathogenicity of the organism (Nakano et al., 2012). *Salmonella* will continue to be a concern to public health due to its ability to overcome species barriers and adapt to new hosts or environments, as is seen with the ability of *Salmonella* to colonize in lymph nodes (Baumler et al., 1998).

As of 2011, nontyphoidal *Salmonella* was the second most frequent cause of foodborne illness in the United States (CDC, 2014; CDC, 2015a). In more serious scenarios, *Salmonella* was number one—of the number of foodborne illnesses that resulted in hospitalization, *Salmonella* accounted for 35% of cases and 28% of deaths (CDC, 2014).

1.6 *Salmonella* and Pet Food

The safety and quality of pet food and treats (i.e., dog and cat products) has become a challenging and complex issue in recent years (Silliker, 2014). The pet food industry is continually growing and is now facing obstacles including a global supply chain, emerging contaminants, and increased regulatory requirements (Silliker, 2014). The safety of pet food is not only of concern for the safety of the animals but also to public health due to direct contact that humans have with the product, thus prompting increased federal oversight (Silliker, 2014). New regulation for the animal feed industry came in the form of the Food Safety Modernization Act (FSMA), which allowed for the Food and Drug Administration (FDA) to implement new requirements that are impacting the daily operations of pet food manufactures and their suppliers (Silliker, 2014). The new law requires facilities that manufacture, process, package, and hold animal food to implement risk-based preventative systems in order to manage potential hazards (Silliker, 2014). This includes a written product safety plan. A preventive plan includes physical, chemical, biological, and radiological hazards, as well as any potential risks to animal health that could result from nutrient deficiencies or excesses (Silliker, 2014). In the new law, animal food is defined as “food for animals other than man, and includes pet food, feed, and raw materials and ingredients” (Silliker, 2014). This includes feed not only for companion pets but also for livestock and other captive animals (Silliker, 2014).

The need for new regulations was driven by a dramatic increase in pet food recalls and import alerts on ingredients received from international sources, as well as the implications to human health (Silliker, 2014). Of the common pathogens associated with human illness, *Salmonella* is the leading cause of recalls of pet food (Silliker, 2014). In 2012, a multistate outbreak of 49 cases of human salmonellosis was linked to multiple brands of dry pet food (CDC, 2012a; Silliker, 2014). The causative agent, *Salmonella* Infantis, was linked back to a single processing plant in Gaston, South Carolina (CDC, 2012a). The strain was isolated from one ill dog and one that was asymptomatic—outbreaks affect both human and pet health (CDC, 2012a). In 2007, the contamination of dry pet food with a rare serovar, *S.* Schwarzengrund, resulted in 79 cases of human salmonellosis across 21 states (Silliker, 2014). The outbreak was traced back to a processing facility in Pennsylvania (CDC, 2007a). In this case, it was probable that the contamination came from the environment; the facility was ordered to temporarily shut down for sanitation (CDC, 2012a). Both large outbreaks involved dry, extruded, 'kibble-type' dog and cat foods.

With FSMA, FDA indicated that environmental monitoring of *Salmonella* is appropriate for facilities that produce pet food (Silliker, 2014). While environmental monitoring is not mandatory, it is thought to be a mitigation strategy for protecting product from coming into contact with *Salmonella* during processing (Silliker, 2014). Environmental monitoring of *Salmonella* is a tool to determine the effectiveness of sanitation, a major preventive control under FSMA. However, the FDA will be enforcing a 'zero tolerance' policy for *Salmonella* in all finished pet food due to the risk to human and animal health (Silliker, 2014). A 2012 FDA report on the *S.* Infantis outbreak indicated that the contamination could have come from incoming animal fats and oils due to a lack of microbiological testing prior to use (FDA, 2012). Pets may

become infected from consuming contaminated food, asymptomatically or clinically, thus increasing the risk for human exposure (CDC, 2013; FDA, 2013b). Human handling of pet food presents an additional concern if product is contaminated with *Salmonella*. Risks are increased for children, who often are in closer contact with pets and their food, and elderly as well as those with a weakened immune system (CDC, 2013). The same standards are not required for livestock or other animal feeds due to the low risk to humans (FDA, 2013a). For these products, regulations are only concerned with strains of *Salmonella* that would cause illness in the animal (FDA, 2013a; Silliker, 2014).

1.7 *Salmonella* Growth Characteristics

Salmonella presents a unique hazard and significant challenge to human and pet food producers due to its ability survive in high fat, low moisture matrices (Humphrey, 2004; Finn et al., 2013; Li et al., 2014). Peanut butter, a matrix with these qualities, has been implicated in numerous outbreaks in recent years. The first documented outbreak from peanut butter was in 1996, but since 2006, at least 3 outbreaks of *Salmonella* in peanut butter have occurred (Li et al., 2014). Other low moisture products that have been associated with *Salmonella* outbreaks include chocolate, cereal, nuts, powdered milk, black pepper, and dried dog food (Finn et al., 2013). It has been shown that these types of matrices protect bacterial cells, thus limiting the effectiveness of heat treatment (Humphrey, 2004; Li et al., 2014). Ma et al. (2009) demonstrated that longer exposure at higher temperatures was needed for sufficient lethality of *Salmonella* in peanut butter. *Salmonella* has been shown to survive throughout the food chain due to its effective response to environmental changes and its ability to make changes to both its phenotype and genotype in response to exposure to environmental conditions (Humphrey, 2004). Repeated exposure to a stressor, especially at sub-lethal levels, will allow bacterial cells to better tolerate

stressors (Humphrey, 2004). He et al. (2011) demonstrated that stressed *Salmonella* cells exhibited less susceptibility to heat treatment than fresh cells likely due to cross-protection. Bacteria cells that are less susceptible to heat have increased pathogenicity, being able to cross the gastric acid barrier in infected hosts with greater ease (Humphrey, 2004; Finn et al., 2013).

It was previously believed that matrices with decreased water activity and moisture levels would inhibit the growth and perhaps the survival of *Salmonella* (Humphrey, 2004; Finn et al., 2013). However, it is now understood that under a decreased water activity condition, growth may continue and multinucleate filaments may form—this has been observed with both *S.* Typhimurium and *S.* Enteritidis (Humphrey, 2004). Under laboratory conditions, once cells are removed from a low water activity matrix, cell division and replication occurs rapidly (Humphrey, 2004). This could have a large impact on public health and food safety—if a food product was tested immediately following a storage period where cells were able to grow and form filaments, the bacterial count would be low; but, once that product returned to favorable conditions, bacterial cell growth would occur rapidly (Humphrey, 2004).

Often times when bacteria are adapted to a harsh environment, they develop tolerance to other stressors via cross-protection. When *Salmonella* were exposed to a low water activity environment, increased thermal tolerance was observed (Finn et al., 2003). It is generally accepted that moisture aids in destruction of organisms thus a lack of moisture would allow for decreased susceptibility of cells to heat treatment (Finn et al., 2003). Another feature of high fat, low moisture foods is the common observation of non-linear death curves, showing a concave-upward curvature, during heat inactivation (Finn et al., 2003). This again indicated the protective nature of high fat, low moisture matrices.

When fats and oil (high fat, low moisture) are recycled, rendered and heat treated, any bacterial contamination may be able to gain tolerance to heat due to prolonged exposure at sub-lethal temperatures. Continuous exposure, perhaps at multiple time periods, in combination with the protective nature of the matrix, may decrease the heat susceptibility of bacteria (He et al., 2011). In the 2012 outbreak caused by *S.* Infantis in dry pet food, it was considered highly likely that the addition of rendered animal fats and oil was the source of contamination (FDA, 2012). It is believed that the ability of *Salmonella* to adapt to environmental changes has caused a high variability in infective dose (Humphrey, 2004; Finn et al., 2013).

1.8 *Escherichia coli* O157:H7

Theodor Escherich first described *Escherichia coli* in 1885 (Todor, 2008; Lim et al., 2010). The organism was isolated from the feces of newborns and named *Bacterium coli commune,* later to be renamed *E. coli* (Todor, 2008). For many years, the organism was considered to be commensal bacteria of the large intestine—it was not until *E. coli* was identified as the cause of a diarrheal outbreak among children in the 1930s that it was known to cause illness in humans (Todor, 2008). While most *E. coli* remains a commensal organism in the intestinal tract, strains have evolved to become pathogenic to humans due to the acquisition of virulence factors (Lim et al., 2010). Shiga-toxin producing *E. coli*, known as enterohemorrhagic *E. coli* (EHEC or STEC), pose the greatest concern to human health (FDA, 2012). Serotype O157:H7 accounts for 75% of EHEC infections, but other serotypes (known as the "big 6") are emerging as causative agents of foodborne illness: O111, O26, O121, O103, O145, and O45 (FDA, 2012). This group of serotypes also is referred to as Shiga toxin-producing *E. coli* (STEC; CDC, 2015c). Serotypes are named based on the phenotype of both the O-group and the flagellar structure—thus *E. coli* O157:H7 expresses the somatic (O) antigen 157 and the flagella (H)

antigen 7 (Mead and Griffin, 1998; Lim et al., 2010). *E. coli* O157:H7 was first identified as a human foodborne pathogen when it was determined to be the cause of an outbreak of bloody diarrhea in Oregon and Michigan in 1982 linked to hamburgers (Lim et al., 2010).

E. coli is part of the large bacterial family *Enterobacteriaceae* which is comprised of gram-negative, rod-shaped, facultative anaerobes (Todor, 2008; Lim et al., 2010). Pathogenic *E. coli* is categorized based on virulence factors, clinical symptoms, mechanisms, serogroups, or pathogenicity (Lim et al., 2010). Among pathogenic strains, enterohemorrahagic *E. coli* produce Shiga toxins (Stx) that cause hemorrhagic colitis and hemolytic uremic syndrome in humans (Lim et al., 2010). While occurrence of *E. coli* O157:H7 as the causative agent for illness is lower than other human pathogens such as *Salmonella*, the illness caused by the organism is much more severe, resulting in higher hospitalization and death rates (Lim et al., 2010). Most cases start with non-bloody diarrhea and are self-limiting, but some patients can progress to bloody diarrhea and hemorrhagic colitis within 1 to 3 days (Lim et al., 2010). Hemorrhagic colitis is characterized by bloody diarrhea, extreme abdominal cramps, and little-to-no fever (Mead and Griffin, 1998). In 5 to 10% of hemorrhagic colitis patients, the disease further progresses to hemolytic uremic syndrome, which is life-threatening, especially for children and the elderly (Lim et al., 2010). Hemolytic uremic syndrome (HUS) causes acute renal failure, hemolytic anemia, and low platelet count (Tarr et al., 2005). The mortality rate of HUS is around 5%, but survivors are likely to have permanent disabilities such as renal insufficiencies and neurological deficits (FDA, 2012).

Shiga toxins (Stx) produced by EHEC serotypes are responsible for causing illness and are divided into two groups—Stx1 and Stx2 (Lim et al., 2010; FDA 2012). Virulent isolates may express Stx1, Stx2, or both (Lim et al., 2010; FDA 2012). The Stx2 gene is more commonly

associated with hemorrhagic colitis and hemolytic uremic syndrome (Lim et al., 2010; FDA, 2012). Virulent EHEC serotypes also carry the 'locus of enterocyte effacement' (LEE) which is a pathogenicity island that encodes for intimin, a protein required for bacterial attachment to epithelial cells (FDA, 2012). *E. coli* O157:H7 colonizes the intestinal mucosa and causes lesions, characterized by effacement of microvilli and bacterial attachment to epithelial cell membrane via LEE-encoded factors (Lim et al., 2010; FDA, 2012). Cells then produce Stxs are internalized, activated, and can pass into the bloodstream to become systemic (FDA, 2012).

In the United States, the most common route of transmission is via consumption of contaminated food with the infective dose ranging from 10 to 100 cells (Lim et al., 2010; FDA, 2012). The organism can be spread via human contact with human or animal feces (Lim et al., 2010). Symptoms typically begin within 4 days of exposure, but can range up to 9 days (FDA, 2012). The average duration in uncomplicated cases is 8 days (FDA, 2012). Treatment for *E. coli* O157:H7 infections is mainly supportive (Mead and Griffin, 1998). The use of antibiotics to treat the infection is controversial due to the possible increased risk for developing HUS after receiving treatment (Meat and Griffin, 1998). Cattle are a common asymptomatic reservoir for the pathogen—human cases have been reported from coming in contact with cattle at petting zoos, dairy farms, and campgrounds (Lim et al., 2010). Since cattle are natural carriers of the pathogen, *E. coli* O157:H7 is of great concern during beef slaughter. In 2015, beef accounted for 46% of illnesses caused by *E. coli* O157 while vegetable row crops contributed to 36% of illnesses caused by the pathogen (IFSAC, 2015). According to the Centers for Disease Control and Prevention, there are approximately 63,000 cases of EHEC infections each year in the United States (FDA, 2012).

E. coli O157:H7 has been shown to survive and adapt to varied environments, including extreme temperature or pH changes (Lim et al., 2010). The organism has been shown to persist in both predictable and unusual environments for notable lengths of time (Beauchamp and Sofos, 2010). Extrinsic and/or intrinsic factors may affect the heat or acid tolerance of the organism (Ahmed et al., 1995). Ahmed et al., (1995) found that as fat content of meat products increased, so did the number of surviving *E. coli* O157:H7 populations. The high fat content likely worked to protect cells from heat destruction (Ahmed et al., 1995; He et al., 2011). However this phenomenon has been seen with many pathogens, not just *E. coli* O157:H7 (Ahmed et al., 1995).

A more unique characteristic of *E. coli* O157:H7 is its ability to survive in acidic foods (Ryu and Beuchat, 1998). Acidification is a common control mechanism to control growth or to kill pathogenic organisms in or on food, but *E. coli* O157:H7 has been shown to resist inactivation in apple cider, mayonnaise, and fermented meats (Ryu and Beuchat, 1998). Both acid-adapted and acid-tolerant bacterial cells are a concern for food processors (Ryu and Beuchat, 1998). As has been seen with other bacteria, once *E. coli* O157:H7 becomes acid-adapted, cross-protection genes are activated thus increasing the organism's ability to resist thermal stress (Leyer et al., 1995; Ryu and Beuchat, 1998). When comparing acid-adapted and acid-shocked cells to a control group, acid-adapted cells showed increased thermal resistance (Ryu and Beuchat, 1998).

1.9 D- and z-values

Many factors influence pathogen death including temperature, pH and inherent characteristics of the specific matrix (fat content, water activity, etc.). With so many variables, the food industry needed a universally accepted standard for determining proper length of heat

exposure to achieve desired microbial reductions (Revox Sterilization Solutions, 2014). A D-value, or decimal reduction time, has become the established standard and is the time (traditionally expressed in minutes) required at a specific temperature to kill 90% (or 1 log) of the organism of concern (Revox Sterilization Solutions, 2014). D-values are determined by conducting heat challenge studies—every matrix is different and, thus, D-values calculated for one matrix cannot be applied to others. Additionally, D-values are specific for the target organism. For example, calculated D-values for *Salmonella* in a beef patty cannot be used for *E. coli* O157:H7 in a beef patty. Similarly, D-values for *Salmonella* in a beef patty cannot be used for *Salmonella* in a pork chop. In a heat challenge study, inoculated samples are heat treated and removed at pre-determined time points (Ahmed et al., 1995). Surviving populations are plotted vs heating temperature, generating a death or survivor curve. Linear regression, when appropriate, is performed to obtain the best-fit line and its slope—the negative reciprocal of this slope is the D-value (Ahmed et al., 1995). It is assumed that heat treatment for the determined exposure time (D-value) at the specific temperature will assure target lethality (Juneja et al., 2001). While linear regression has traditionally been used, it has proven more common for non-linear death curves to be observed, especially in high fat matrices (Juneja et al., 2001). Inactivation models should be generated when non-linear curves are observed, but simply utilizing the linear portion of the curve also is beneficial due to the ease of others replicating the procedure (Juneja et al., 2001). The linear portion of the line must be properly identified—common methods are spline and non-linear regressions (Juenja et al., 2001).

A z-value is the change in temperature (°C) necessary to decrease (or increase) the D-value by a factor of 10 (Forsythe, 2010). Z-values are calculated by plotting D-value vs temperature and performing linear regression to obtain the slope of the best fit line. The negative

reciprocal of the slope is the z-value (Ahmed et al., 1995). Z-values are presented as degree Celsius.

1.10 Preventive Controls

A mandatory element under FSMA is that any facility that manufactures, produces, processes, or holds food for animals must develop and implement a written food safety plan that involves a hazard analysis and establishment of preventive controls (PCs) (FDA, 2015; FDA, 2016b). The ultimate goal of PCs is to minimize or prevent a hazard—this can be at a critical step in the process or whenever control is needed for product safety (FDA, 2016a; FDA, 2016b). Preventive controls have management requirements that include monitoring, corrective actions, verification, and record keeping (FDA, 2015). The FDA requires that preventive controls be based in science—PCs must be scientifically validated prior to use and whenever reevaluation is necessary (typically every 3 years or when a deviation occurs; King and Ades, 2015; FDA, 2016a). It is often required that facilities conduct their own challenge studies to determine appropriate PCs or parameters within PCs (King and Ades, 2015). Preventive controls are often associated with processing parameters such as time, temperature, or pH. Parameters are designed to target a specific hazard and, thus, require scientific validity to ensure that the hazard is adequately under control (King and Ades, 2015). The FDA states that the goal with establishing PCs is to reduce the occurrence of contaminated animal feed thus reducing risks to animal and human health (FDA, 2016a).

CHAPTER 2

Summary

Objectives of this study were to (i) understand lethality of a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat-treated, recycled used cooking oil (UCO), (ii) estimate D- and z-values for a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat-treated, recycled UCO, and (iii) establish time/temperature processing steps targeting a selective set of strains of *Salmonella* and *E. coli* O157:H7 for use as preventive controls in a UCO product safety plan. The objectives were addressed by inoculating previously heat-treated, UCO (high fat), with a five-strain mixture of *Salmonella* or *E. coli* O157:H7, at approximately 8.0 log CFU/ml. Inoculated samples were heat treated at 62, 71, or 82°C using a water bath. At predetermined time intervals, individual samples were removed from water bath and immediately chilled in an ice bath to stop the heating process. Each pathogen and temperature combination was replicated six times. Thermal death curves were generated from the surviving populations (log CFU/ml). Non-linear death curves were observed for all pathogen and temperature combinations, indicative of pathogen survival. Segmented regression was used to calculate D-values, ranging from 0.03 to 0.04 min at 82°C, 0.14 to 0.27 min at 71°C, and 0.77 to 1.49 min at 62°C. Z-values ranged from 14.14°C to 27.78°C. Using this information, appropriate preventive controls were established to create timed heat treatment processes for use by processors of UCO to comply with Food and Drug Administration (FDA) regulations.

Introduction

In the United States, the by-product rendering industry is regulated by the US Food and Drug Administration (FDA) and with the implementation of the Food Safety Modernization Act (FSMA; FDA, 2011), the industry will be examined even more closely to ensure product safety. In this study, a worst-case scenario seen in the rendering industry was developed by studying a high-fat matrix in combination with a selective set of strains known for their persistence and tolerance to extrinsic factors such as heat. Used cooking oil (UCO) is a rendered, recycled, high-fat product that has tremendous application in the pet and animal feeding industries. When used in animal feeds, UCO, also known as yellow grease, serves as a high fat feed ingredient that provides concentrated dietary energy while also enhancing palatability (Meeker and Hamilton, 2006; Nelles et al., 2008). In livestock production, it is used as a finishing period feed ingredient and in companion animal feeds, it is sprayed on dry, extruded, "kibble-type" foods (Nelles et al., 2008).

Outbreaks of human disease are rarely linked back to livestock or pet feed (US General Accounting Office, 2000; Crump et al., 2002; Finley et al., 2006). Nonetheless, as humans are in close proximity to their pets, any contamination on pet food poses a reasonable risk to humans, especially high risk groups such as children, elderly, or the immunocompromised (Crump, 2002; Finley, 2006; Cochrane, 2016). For this reason, the microbial safety of products used in the formation of pet foods is imperative. So much so, that FSMA will actively provide surveillance of the safety of animal feeds and pet food with regulatory bodies expecting renderers to have product safety measures in place, in the form of preventive controls, to ensure destruction of pathogens during processing (Crump et al., 2002; FDA, 2016a; FDA, 2016b).

Bacterial contamination of UCO, specifically by *Salmonella* and *Escherichia coli* O157:H7 presents a safety hazard not only to the animals that consume it, but also to humans handling the product (Crump et al., 2002, Cochrane et al., 2016). When added to animal feeds, UCO does not typically receive any further intervention steps to eliminate bacterial contamination. Furthermore, FSMA and the FDA mandated zero tolerance of *Salmonella* in pet food, thus making the heat treatment of UCO by renderers a critical step in the prevention of bacterial contamination (Carrion and Thompson, 2013).

In order for the rendering industry to comply with new regulations, scientific guidance for rendering processes is a critical need. Specifically, calculation of pathogen reduction due to time and temperature parameters is fundamental in determining accurate, validated processing steps. To facilitate the development of these processing steps, determination of D- and z-values is often used as a tool for predicting pathogen lethality during thermal processing (Murphy et al., 2004). A microbiological D-value is defined as the time required at a specific temperature to reduce the microbial population by 90%, or one log unit whereas a z-value represents the change in temperature required to change the D-value by a factor of 10 (Juneja et al., 2001; Murphy et al., 2004). Objectives of this study were to (i) understand lethality of a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat-treated, recycled UCO, (ii) estimate D- and z-values for a selective set of strains of *Salmonella* and *E. coli* O157:H7 in heat-treated, recycled UCO, and (iii) establish time/temperature processing steps targeting a selective set of strains of *Salmonella* and *E. coli* O157:H7 for use as preventive controls in a UCO, product safety plan.

Materials and Methods

Pre-experimentation work. Limited methodologies are available for the microbiological analysis of high fat and/or oil matrices. Therefore, we established appropriate methodologies to

meet objectives in a laboratory setting. The focus of this pre-experiment work was to refine methodologies in order to most accurately depict microbiological activity in an oil sample.

It is typical in inoculum preparation procedures to resuspend washed cells in phosphate buffered saline (PBS); however, for this particular study, there was concern that cells suspended in an aqueous liquid, such as PBS, would not adequately mix with the UCO sample. Furthermore, addition of an aqueous liquid to the UCO would alter the heat transfer properties, as well as water activity, of the oil. Altering the water activity would not only influence D- and z-values, but also potentially favor heat destruction of bacteria (He et al., 2011; Li et al., 2014). As the influence of these variable is unknown, inoculum cells were resuspended in sterilized, warmed (37°C) vegetable oil (soybean oil; Kroger, Cincinnati, OH) rather than PBS. No difference in microbial populations was observed when an aqueous solution and sterile vegetable oil were compared (data not shown). This corresponds with previous research demonstrating that resuspension in an oil versus aqueous solution is preferred when working with a low moisture, high fat matrix (He et al., 2011; Li et al., 2014).

Furthermore, as expected, preliminary trial work demonstrated that using 0.1% buffered peptone water (BPW; Difco, Sparks, MD) as the diluent resulted in almost immediate separation of the oil and aqueous phases after vortexing. Subsequent dilutions were inaccurate (i.e., a ten-fold reduction of bacterial counts was not obtained in serial dilutions). In order to mitigate the inconsistencies caused by phase separation, we examined the utilization of an emulsifier (Tween80; Acros Organics, Waltham, MA) in the diluent. When 1% Tween80 was included in the diluent, we observed enhanced emulsification and reduced dilution errors—resulting in a more consistent dilution series. Thus, 1% of Tween80 was incorporated into 0.1% BPW for formation of the diluent media (BPW-Tween).

Other pre-experimentation augmentations included the utilization of mass, rather than volume, for all microbiological work. Similarly, we observed the utilization of a larger dilution vessel (50 ml conical tube; Faclon; Fisher Scientific, Waltham, MA) facilitated more thorough vortexing and emulsification when compared to smaller vessels. Overall, the observations and modifications made in pre-experiment work proved useful in refining methodologies prior to the initiation of the project.

Finally, preliminary testing was conducted to determine background microbial contamination as well presence or absence of *Salmonella* and *E. coli* O157:H7. Presence was determined using the methodology described in the microbiological analysis subsection. Out pre-experiment microbial evaluation indicated that *Salmonella* and *E. coli* O157:H7 were not present in the UCO and background microbial contamination was low to not present.

Used cooking oil procurement and analysis. Rendered, recycled, heat treated UCO (n = 2 batches obtained on two separate production days) was obtained from a commercial rendering facility and transported to Colorado State University (Fort Collins, CO). Two samples from each batch were analyzed for fat and moisture content as well as water activity, while the remaining samples were used for the inoculation challenge. Total lipid was determined using a variation of the chloroform:methanol method described by Folch et al. (1957). Moisture was determined using AOAC oven drying methods 950.46 and 934.01 (AOAC International, 1995). Water activity was determined using a water activity meter (Aqualab model series 3, Pullman, WA). Batch 1 averaged to 9.85% moisture, 80.30% fat, and a water activity of 0.895. Batch 2 averaged to 0.30% moisture, 92.35% fat, and a water activity of 0.673.

Pathogen strain selection. Used cooking oil samples were inoculated with a five-strain mixture of *Salmonella* or a five-strain mixture of *E. coli* O157:H7. The *Salmonella* mixture

(Table 1) consisted of two isolates of bovine origin (*Salmonella* Typhimurium DT104 var. Copenhagen and *Salmonella* Newport), two isolates of poultry origin (*Salmonella* Enteritidis and *Salmonella* Heidelberg), and one well-documented heat resistant strain (*Salmonella* Senftenberg 775W [ATCC 43845]). The *E. coli* O157:H7 mixture (Table 1) consisted of four isolates of bovine feces origin (C1-072, C1-109, C1-154, C1-158) and ATCC 43895, isolated from raw hamburger meat. The selected strains are known for their tolerance and persistence and were chosen for this study to create a worst-case scenario (Angelotti et al., 1960; Ng et al., 1969; Carlson et al., 2009).

Inoculum preparation. Working independent cultures of the *Salmonella* and *E. coli* O157:H7 strains were maintained on xylose lysine deoxycholate (XLD; Acumedia, Neogen Corp., Lansing, MI) agar or sorbitol MacConkey (SMAC; Difco) agar, respectively. Before each replication, a single colony of each strain was separately inoculated into 10 ml of tryptic soy broth (TSB; Difco) and incubated at 35°C for 22 ± 0.5 h. Broth cultures were then subcultured by transferring 0.1 ml of the original culture into 10 ml of fresh TSB. After incubation (35°C, 22 ± 0.5 h), broth cultures of the five *Salmonella* or five *E. coli* O157:H7 strains were combined (100 ml total per pathogen) and cells were harvested by centrifugation (3,220 × *g*, 20 min, 4°C; Eppendorf model 5810R, Brinkmann Instruments Inc., Hamburg, Germany). Supernatant was discarded and cell pellets were washed with 10 ml of phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich, St. Louis, MO). Cell suspensions in PBS were re-centrifuged as previously described, the supernatant was discarded, and cells were resuspended in 10 ml of pre-warmed (37°C) sterilized vegetable oil. This tenfold concentration of the inoculum suspension allowed for an inoculation level of approximately 8 log CFU/ml when 0.2 ml of inoculum was added to

4.8 ml of used cooking oil (total volume of 5 ml). This high starting inoculation level was used in order to create a worst-case contamination scenario.

Heat challenge studies. A total of six replications, performed on six separate days, were conducted for each pathogen and temperature combination. The inoculated UCO samples were evaluated at three different temperatures—62, 71, and 82°C. On the initiation of each replication, 4.8 ml of UCO was distributed into sterile borosilicate glass test tubes (16 × 150 mm; Fisher Scientific). Heat challenges were performed using a water bath (Precision Scientific, Shaking Water Bath 50, Waltham, MA) fitted with a circulator (Fisher Scientific, Model 73, Pittsburg, PA). Water level in the bath was maintained at 4 to 5 cm above the level of the UCO samples. Before initiation of heat treatments, water was pre-heated to the desired temperature and allowed to stabilize for a minimum of 15 min. Simultaneously, UCO samples were equilibrated to the desired temperature (i.e., 62, 71, or 82°C). The temperature of the water bath and two tubes of used cooking oil (located on either end of the water bath) were continuously monitored using Type K thermocouples (Pico Technology Ltd., Cambridge, UK).

Before to inoculation, the cocktail mixture of bacterial strains was vortexed and visually inspected for homogeneity. Oil samples (n = one sample per time interval) were removed from the water bath and immediately inoculated with 0.2 ml of either the *Salmonella* or *E. coli* O157:H7 inoculum. Inoculated samples were immediately vortexed for 3 s and placed back into the water bath. Timed sampling intervals (Table 2) were determined before experimentation and selected in order to generate survivor curves capturing a full-range of cell death. At the completion of each time interval, the appropriate samples were removed from the water bath and immediately placed in an ice/water slurry (0°C ± 5°C) for a minimum of 5 min before microbial

analysis to inhibit residual heating. The ice/water slurry depth was maintained 7 cm above the oil sample.

Microbiological analysis. Dilution blanks (9 ml) used for performing tenfold serial dilutions of the heat treated and non-heat treated (0 min or 0 s time intervals) samples were comprised of 0.1% BPW supplemented with 1% Tween 80 warmed to 37°C. Vortexing of the first sample dilution was standardized to 30 s (50-ml Falcon Tube).

Appropriate dilutions of oil samples were surface-plated, in duplicate, onto both selective and non-selective recovery agars. Xylose-lysine deoxycholate (XLD) served as the selective medium for *Salmonella* spp., SMAC served as the selective medium for *E. coli* O157:H7, and tryptic soy agar (Acumedia) supplemented with 1% sodium pyruvate (Fisher Scientific; TSAP) served as the non-selective recovery medium. The selective agars promoted the growth of their intended pathogen survivors (i.e., non-pathogenic background flora and non-viable pathogens were prohibited from growth) while the TSAP allowed for growth of both survivors and heat injured cells. Selective agar plates were incubated for 48 h at 35°C while TSAP plates were incubated for 72 h at 25°C.

Plating of undiluted UCO affected accurate recovery of surviving populations. The undiluted used cooking oil formed a thick film on the agar and inhibited the formation of colonies. Thus, undiluted samples were not plated and a detection limit of 10 CFU/ml (1 log CFU/ml) was established.

Statistical analysis, D-value, and z-value calculations. D-values are determined by plotting log CFU/ml of survivors versus time and are traditionally calculated using simple linear regression. However, in the current project, surviving populations of pathogens exhibited a tailing effect as heat-exposure time increased. Previous research studies have also demonstrated

non-linear death curves in some microbial populations (Juneja et al., 2001). To accommodate the lack of linearity, segmented (piecewise) regression was performed using the NLIN Procedure in SAS (v 9.4; Cary, NC). This procedure separated the data into two portions: an initial segment, showing a greater magnitude of linear reduction (ranging from a 3 to 6 log reduction) and a second segment exhibiting the tailing of survivors. When splitting the data, the NLIN procedure estimated cut points for each pathogen, time, temperature, and agar combination (i.e., each combination was graphed and estimated separately). Cut points were determined by SAS based on the median time point; the system generated iterations of the cut points until the best fit parameters were identified. The initial linear section, which produced a greater than 90% reduction of initial populations, was used to calculate D-values. Specifically, the negative reciprocal of the slope obtained from the segmented regression line of the initial segment was used for D-value calculations: D = - [1/slope]. Z-values were obtained by plotting the D-values versus process temperatures. Linear regression was used to establish a best fit line and the negative reciprocal of that line was used to determine the z-value: z = - [1/slope].

Results and Discussions

Thermal death curves. Thermal death curves were generated by graphing log CFU.ml of survivors versus time. A noticeable tailing of survivors (i.e. initial decrease of bacterial populations with a low level of persisting survivors over time) was observed for all strain mixtures across all temperatures and agars (Ma et al., 2009; Figures 1 through 6). Although our detection limit was 1 log CFU/ml, surviving populations often persisted above this, some as high as 5 log CFU/ml following heat treatment. In some cases, for example *E. coli* O157:H7 at 62°C, there was a large difference between survival counts on TSAP and on the selective agar, SMAC. The TSAP medium allows for recovery of sub-lethally injured cells. The large difference in

counts between agars indicates the possibly of bacterial cells being sub-lethally injured and, when given the proper environment, be able to persist. This non-uniform destruction and evidence of pathogen survival following extensive heat treatment demonstrated the ability of these selected strains to persist after heat exposure. While the strains used in this study are known to be heat tolerant, previous research has demonstrated the insulation of bacterial cells in a high fat matrix (Angelotti et al., 1960; Juneja et al., 2001; Ma, et al., 2009). Persistence of pathogens following heat treatment in high fat matrices has also been observed (Ma, et al., 2009; Li, et al., 2014). For example, peanut butter is a low moisture, high fat matrix that has been implicated in multiple, nationwide outbreaks of salmonellosis (CDC, 2007; Ma, et al., 2009; CDC, 2012). Previous work has demonstrated that a high fat, low moisture combination promotes increased thermal resistance of pathogens such as *Salmonella* (Li, et al., 2014). In the rendering industry, heat treatment could be utilized as a simple and cost effective intervention, thus, the persistence of pathogens to this process poses a challenge. Further investigation of the mechanisms of pathogen persistence in the presence of heat in a high fat matrix is warranted.

D- and z-values. D- and z-values were calculated from the pathogen populations recovered after heat treatment using selective and non-selective recovery media. The non-selective recovery agar, TSAP, allows for the recovery of heat damaged cells, thus it most accurately represents a situation in which heat damaged cells may exist. D-values for pathogen populations recovered using TSAP, SMAC, and XLD are presented in Tables 3. Z-values for all pathogens are shown in Table 4. The D-values represent the time required at the given temperature to achieve a 1-log CFU/ml reduction in the selected mixture of strains used in this study. Although no studies have evaluated D-values for UCO, determination of D-values at similar temperatures for other high fat matrices (i.e. peanut butter) noted high D-values (He et

al., 2011). In previous research when *E. coli* O157:H7 and *Salmonella* were compared, survival rates were significantly different, with *E. coli* O157:H7 being more susceptible to thermal treatment (He et al., 2011). In this study, both pathogens had similar susceptibility to heat treatment and thus similar D-values. Stopforth et al. (2008) calculated lower z-values for *Salmonella* than were found in this study—z-values ranged from 6.2°C to 7.8°C. This is not unusual as z-values are calculated based upon D-value. Stringer et al. (2000) performed a literature review on thermal inactivation of *E. coli* O157:H7 and found that with high moisture foods such as red meat and apple juice, D-values at 60°C ranged from 0.8 min to 1.9 min. Z-values ranged from 5.5°C to 10.5°C. Likely due to the higher moisture content and lower fat content of these matrices, D-values were lower to values calculated in this study. It is known that D- and z-values must be calculated for every specific matrix and those determined for one medium cannot be applied to others. For that reason, it is acceptable to see data that varies from previous work with other matrices.

The determined time/temperature processing steps represent the time required at a given temperature to achieve a 5-log CFU/ml reduction in the population of the selected strains used in this study, if present in UCO. Time/temperature processing steps were determined for the temperatures of interest by multiplying the D-value, based on populations recovered with TSAP, by five (Table 5). These recommended time/temperature processing steps can be used by processors of UCO as scientifically validated preventive controls for compliance with FDA regulations within FSMA.

While methodologies were established and objectives were reached, pathogen survival at high temperature exposure suggest further research is warranted. Specifically, the presence of surviving pathogens and the observed tailing effect indicate not only the possibility of pathogen

persistence following exposure to heat treatment, but also the need for validation using procedures that more accurately reflect those used in a rendering facility. The mechanisms by which pathogens are able to withstand high temperature exposure is not understood in the UCO matrix; however it is plausible that the matrix properties provide a unique environment for survival. Furthermore, it is possible that the temperature come-up and reduction time observed during processing in a rendering facility would result in a greater magnitude of pathogen destruction when compared to inoculation of preheated samples as performed in this study (in order to meet objectives). Practical next steps regarding heat treatment of UCO would include in-plant validation. Nonetheless, the recommended time/temperature processing steps established in this study can be used by processors to generate heat treatments to comply with FDA regulations. The D- and z-values generated by this study build upon a body of validated scientific literature for ensuring the safety of rendered, recycled UCO.

Table 1. Serological information for *Escherichia coli* O157:H7 and *Salmonella* spp. strains used to inoculate used cooking oil.

Inoculum	Serotype/Serogroup	Strain ID	Origin	Source
E. coli O157:H7	O157:H7	C1-072	bovine feces	Carlson et al. (2009)
	O157:H7	C1-109	bovine feces	Carlson et al. (2009)
	O157:H7	C1-154	bovine feces	Carlson et al. (2009)
	O157:H7	C1-158	bovine feces	Carlson et al. (2009)
	O157:H7	ATCC 43895	raw hamburger	ATCC[1]
Salmonella	Typhimurium DT104 var. Copenhagen	fed plant 2	cattle hides	Bacon et al. (2003)
	Newport	FSL S5-436	bovine	Cornell University[2]
	Enteritidis	FFSRU SE NN	poultry (GI tract)	USDA-ARS-SPA[3]
	Heidelberg	JAB 13556 SL	poultry (GI tract)	USDA-ARS-SPA[3]
	Senftenberg 775W	ATCC[1] 43845	egg products	ATCC[1]

[1]American Type Culture Collection

[2]Dr. Martin Wiedmann, Department of Food Science, Cornell University, Ithaca, NY

[3] Dr. Thomas Edrington, USDA-ARS-SPA, Food and Feed Safety Research Unit, College Station, TX

Table 2. Sampling time points for rendered, recycled used cooking oil (UCO) inoculated with *Escherichia coli* O157:H7 and *Salmonella* spp. at three temperatures.

Temperature (°C)	Sampling time points
62	0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 9, 12, 15, 20 min
71	0, 10, 20, 30, 40, 50, 60, 90, 120, 180 s
82	0, 5, 10, 15, 20, 25, 30, 45, 60 s

Table 3. Decimal reduction times in min (D-values) for *Escherichia coli* O157:H7 and *Salmonella* spp. in rendered, recycled used cooking oil (UCO) determined using surviving populations recovered with tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or selective media (xylose lysine deoxycholate [XLD], *Salmonella* spp.; sorbitol MacConkey [SMAC], *E. coli* O157:H7).

	D-value[1] (R^2)			
Temperature (°C)	*E. coli* O157:H7		*Salmonella*	
	TSAP	SMAC	TSAP	XLD
62	1.49 (0.82)	0.77 (0.85)	1.34 (0.87)	0.79 (0.87)
71	0.27 (0.75)	0.14 (0.76)	0.25 (0.83)	0.20 (0.77)
82	0.04 (0.72)	0.03 (0.70)	0.04 (0.75)	0.04 (0.74)

[1]Decimal reduction times presented in min

Table 4. Calculated z-values for *Escherichia coli* O157:H7 and *Salmonella* spp. in rendered, recycled used cooking oil (UCO).

	z-value (°C)	
Agar	*E. coli* O157:H7	*Salmonella*
SMAC[1]	27.78	--
XLD[2]	--	27.20
TSAP[3]	14.14	15.72

[1] SMAC: sorbitol MacConkey; selective agar for *E. coli* O157:H7.
[2] XLD: Xylose lysine deoxycholate; selective agar for *Salmonella.*
[3] TSAP: Tryptic soy agar supplemented with 1% sodium pyruvate; non-selective culture medium.
-- indicates the agar is not applicable to the pathogen

Table 5. Recommended time/temperature processing steps (i.e. time [min] required at a given temperature to achieve a 5-log CFU/ml reduction) for selected strains of *Escherichia coli* O157:H7 and *Salmonella* spp. used in this study in rendered, recycled used cooking oil. Time/temperature processing steps are based on calculated decimal reduction times (D-values) from surviving populations recovered with tryptic soy agar supplemented with 1% sodium pyruvate (TSAP).

	Time/Temperature Processing Steps (min)	
Temperature (°C)	*E. coli* O157:H7	*Salmonella*
62	7.46	6.72
71	1.34	1.26
82	0.20	0.20

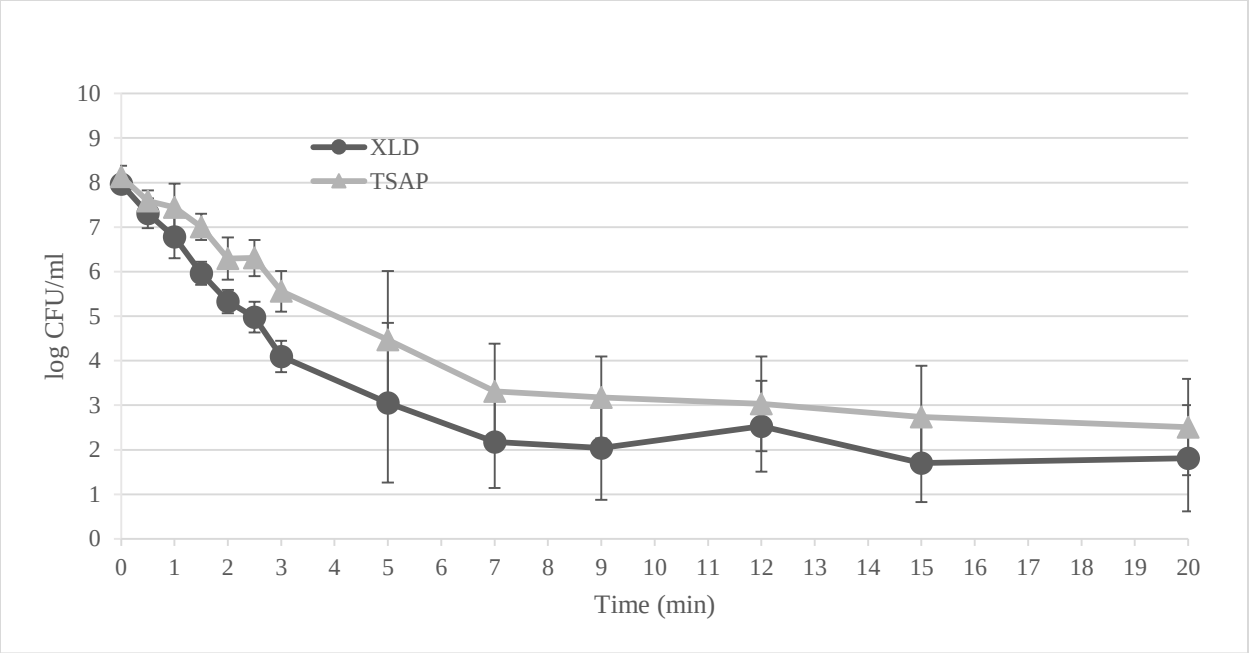

Figure 1. Mean (log CFU/ml) *Salmonella* spp. populations recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or xylose lysine deoxycholate agar (XLD) following heat treatment of inoculated used cooking oil at 62°C.

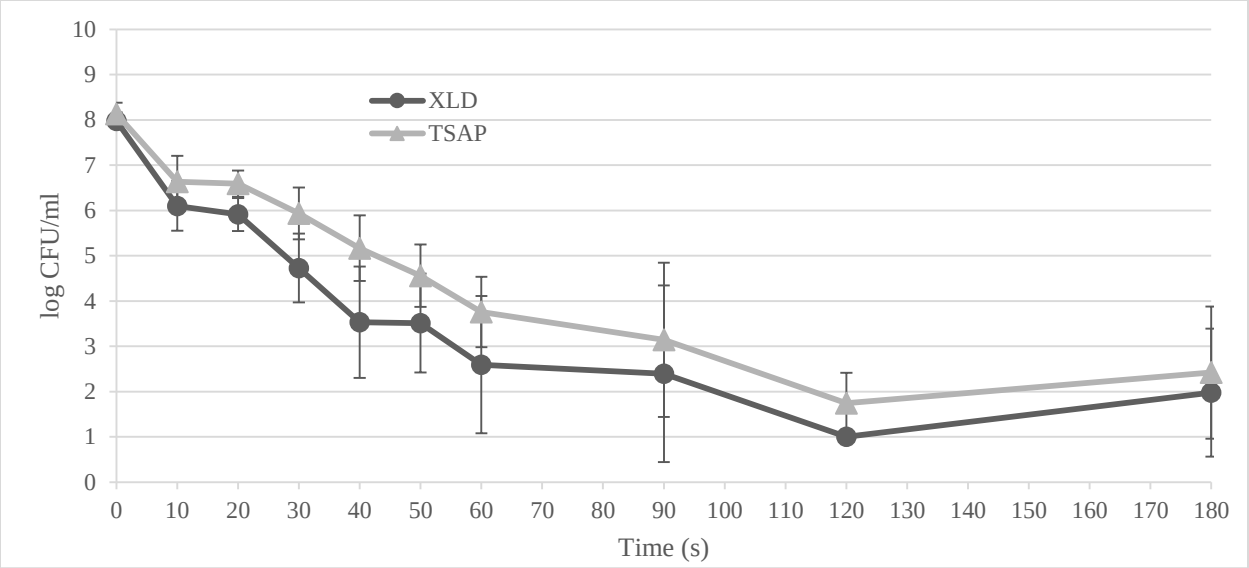

Figure 2. Mean (log CFU/ml) *Salmonella* spp. populations recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or xylose lysine deoxycholate agar (XLD) following heat treatment of inoculated used cooking oil (UCO) at 71°C.

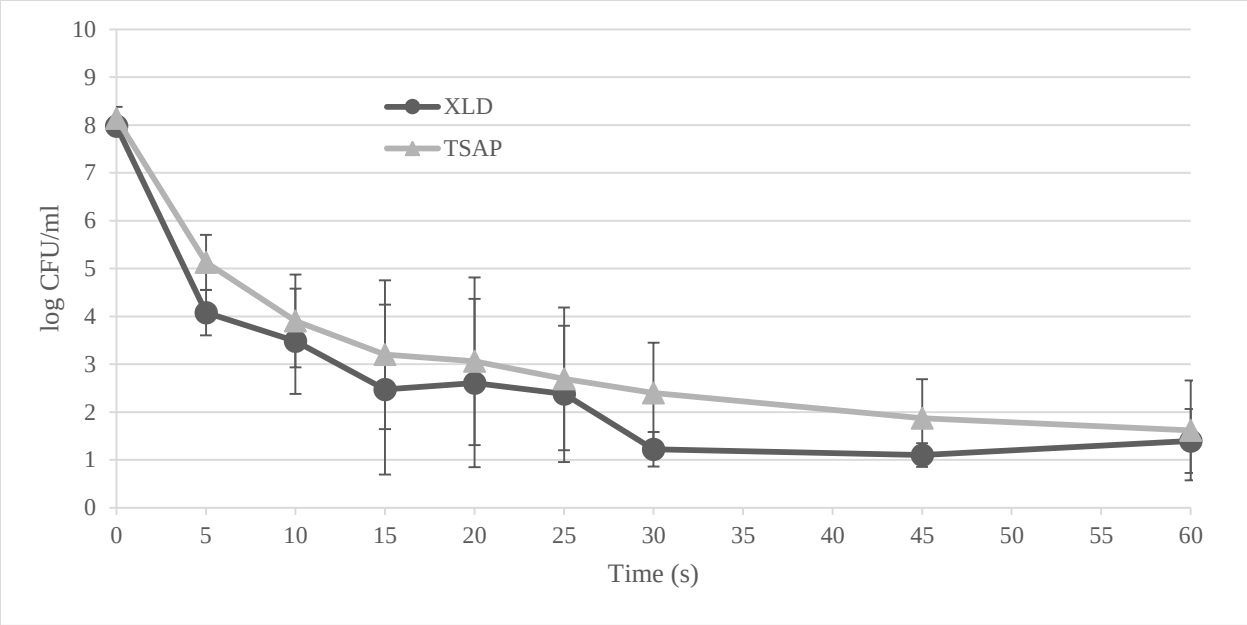

Figure 3. Mean (log CFU/ml) *Salmonella* spp. populations recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or xylose lysine deoxycholate agar (XLD) following heat treatment of inoculated used cooking oil (UCO) at 82°C.

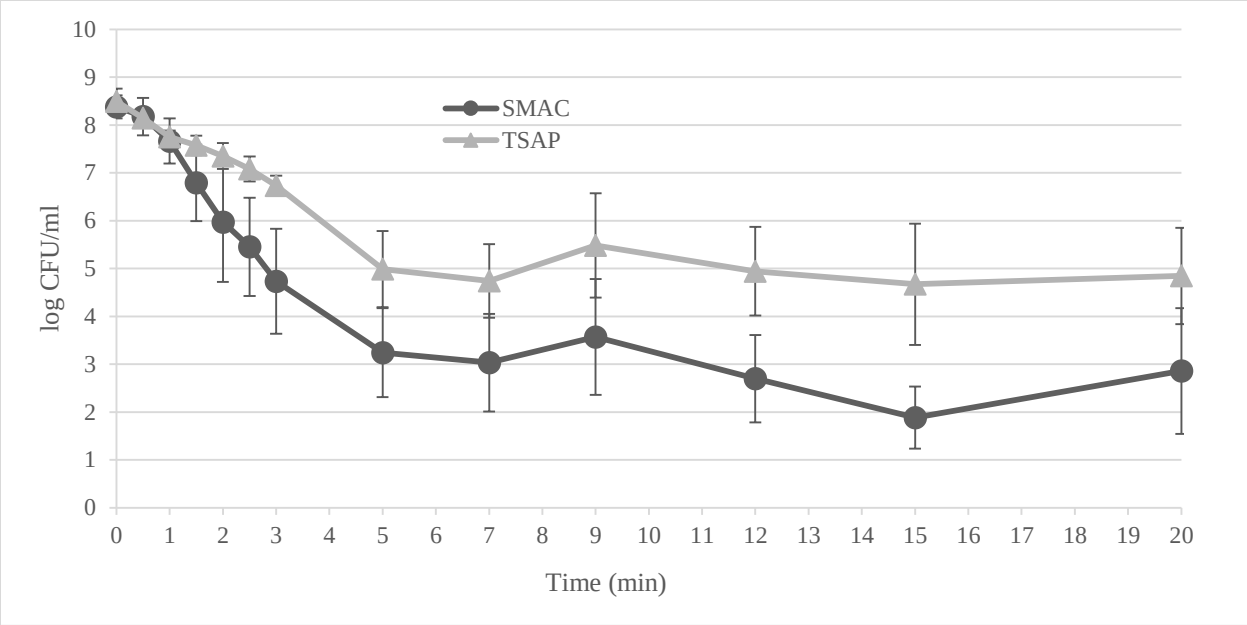

Figure 4. Mean (log CFU/ml) *Escherichia coli* O157:H7 plate counts recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or sorbitol MacConkey agar (SMAC) following heat treatment of inoculated used cooking oil (UCO) at 62°C.

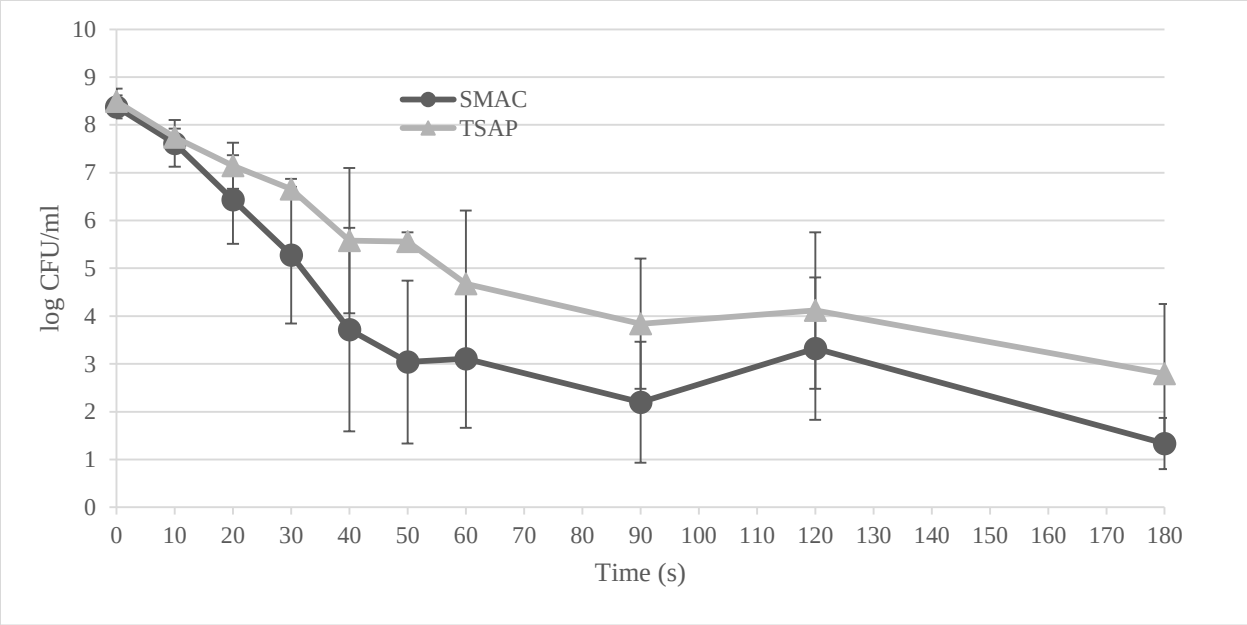

Figure 5. Mean (log CFU/ml) *Escherichia coli* O157:H7 plate counts recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or sorbitol MacConkey agar (SMAC) following heat treatment of inoculated used cooking oil (UCO) at 71°C.

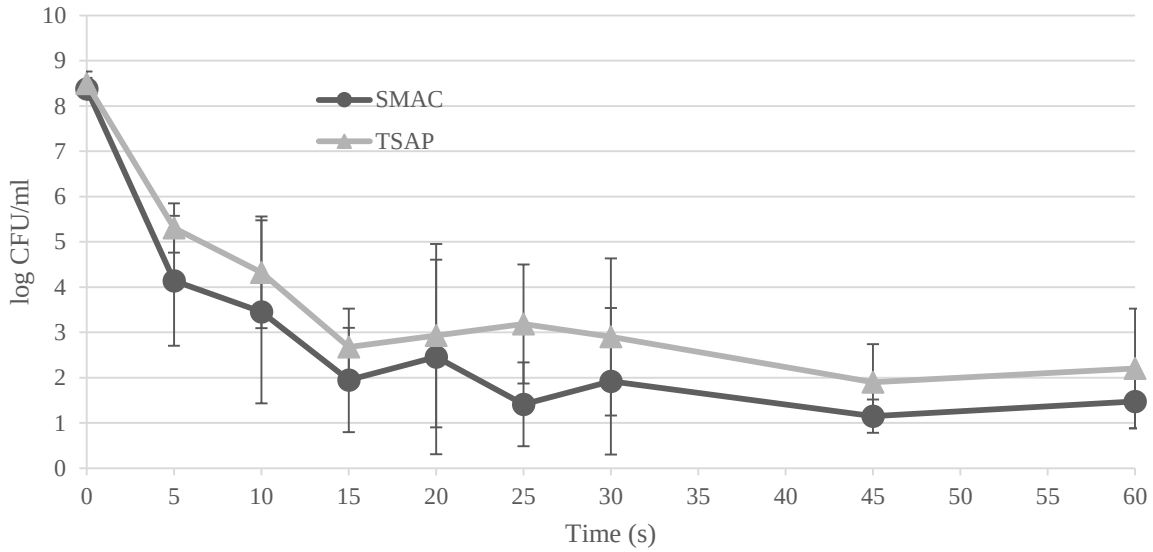

Figure 6. Mean (log CFU/ml) *Escherichia coli* O157:H7 plate counts recovered using tryptic soy agar supplemented with 1% sodium pyruvate (TSAP) or sorbitol MacConkey agar (SMAC) following heat treatment of inoculated used cooking oil (UCO) at 82°C.

REFERENCES

Ahmed, N.M., D.E. Conner, and D.L. Huffman. 1995. Heat-resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. *J. Food. Sci.* 60:606-610.

Aldrich, G. 2006. Rendered products in pet food. *In* D. L. Meeker (ed.), Essential rendering. National Renderers Association, Alexandria, VA. p. 159-177.

American Pet Products Association. 2016. Pet industry market size & ownership statistics. Available at: http://www.americanpetproducts.org/press_industrytrends.asp. Accessed 11 July 2016.

Angelotti, R., M.J. Foter, K.H. Lewis. 1960. Time-temperature effects on Salmonellae and Staphylococci in foods: III Thermal Death Time Studies. 9:308-315.

AOAC International. 1995. Official methods of analysis. Arlington, VA: Association of Official Analytical Chemists.

Bacon, R. T., J. N. Sofos, P. A. Kendall, K. E. Belk, and G. C. Smith. 2003. Comparative analysis of acid resistance between susceptible and multi-antimicrobial-resistant *Salmonella* strains cultured under stationary-phase acid tolerance-inducing and noninducing conditions. *J. Food Prot.* 66:732-740.

Baumler, A.J., R.M. Tsolis, T.A. Ficht, and L.G. Adams. 1998. Evolution of host adaptation in *Salmonella enterica*. *Infect. Immun.* 66: 4579-4587.

Beauchamp, C. S. and J. N. Sofos. 2010. Diarrheagenic *Escherichia coli*. p. 71-94. *In* V. K. Juneja and J. N. Sofos (ed.), Pathogens and Toxins in Foods: challenges and interventions, ASM Press American Society for Microbiology, Washington, DC.

BHT ReSources. 2016. Waste Cooking Oil 101. Available at: http://bhtonline.com/waste-cooking-oil-101/. Accessed 1 May 2016.

Bisplinghoff, F.D. 2006. A history of north American rendering. *In* D. L. Meeker (ed.), Essential rendering. National Renderers Association, Alexandria, VA. p. 1–16.

Carlson, B. A., K. K. Nightingale, G. L. Mason, J. R. Ruby, W. T. Choat, G. H. Loneragan, G. C. Smith, J. N. Sofos, and K. E. Belk. 2009. *Escherichia coli* O157:H7 strains that persist in feedlot cattle are genetically related and demonstrate an enhanced ability to adhere to intestinal epithelial cells. *Appl. Environ. Microbiol*. 75:5927-5937.

Carrion, P.A. and L.J. Thompson. 2013. Pet food, p. 379-396. *In* Y. Motarjemi and H. Lelieveld (ed.), Food Safety Management, 1st Ed. Academic Press, Waltham, MA.

Centers for Disease Control and Prevention (CDC). 2015c. *E. coli* (*Escherichia coli*). Available at: http://www.cdc.gov/ecoli/general/index.html#what-are-shiga-toxin. Accessed 29 May 2016.

Centers for Disease Control and Prevention (CDC). 2014. Estimates of foodborne illness in the United States. Available at: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html. Accessed 18 May 2016.

Centers for Disease Control and Prevention (CDC). 2015a. Foodborne germs and illnesses. Available at: http://www.cdc.gov/foodsafety/foodborne-germs.html. Accessed 10 May 2016.

Centers for Disease Control and Prevention (CDC). 2012a. Multistate outbreak of human *Salmonella* infantis infections linked to dry dog food (final update). Available at: http://www.cdc.gov/salmonella/dog-food-05-12/index.html. Accessed 18 May 2016.

Centers for Disease Control and Prevention (CDC). 2012b. Multistate outbreak of *Salmonella* Bredeney infections linked to peanut butter manufactured by Sunland, Inc. (Final Update). Available at: http://www.cdc.gov/salmonella/bredeney-09-12/. Accessed 10 April 2016.

Centers for Disease Control and Prevention (CDC). 2007a. Multistate outbreak of *Salmonella* Schwarzengrund infections linked to dry pet food. Available at: http://www.cdc.gov/salmonella/2007/pet-food-9-4-2007.html. Accessed 18 May 2016.

Centers for Disease Control and Prevention (CDC). 2007b. Multistate outbreak of *Salmonella* serotype Tennessee infections associated with peanut butter—United States, 2006-2007. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5621a1.htm. Accessed 10 April 2016.

Centers for Disease Control and Prevention (CDC). 2013. Pet food and treats—Tips for keeping people and pets healthy and safe from *Salmonella*. Available at: http://www.cdc.gov/features/salmonelladrypetfood/. Accessed 10 May 2016.

Centers for Disease Control and Prevention (CDC). 2015b. *Salmonella*. Available at: http://www.cdc.gov/salmonella/. Accessed 4 May 2016.

Chan, K., S. Baker, C.C. Kim, C.S. Detweiler, G. Dougan, and S. Falkow. 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar Typhimurium DNA microarray. *J. Bacteriol*. 185: 553-563.

Chopra, A.K., J.H. Huang, X.J. Xu, K. Burden, D.W. Niesel, M.W. Rosenbaum, V.L. Popov, and J.W. Peterson. 1999. Role of *Samonella* enterotoxin in overall virulence of the organism. *Microb. Pathogenesis.* 27:155-171.

Coburn, B., G. A. Grassl and B. B. Finlay. 2007. Salmonella, the host and disease: a brief review. *Immunol. Cell Biol*. 85:122-118.

Cochrane, R.A., A.R. Huss, G.C. Aldrich, C.R. Stark, and C.K. Jones. 2016. Evaluating chemical mitigation of *Salmonella* Typhimurium ATCC 14028 in animal feed ingredients. *J. Food. Prot*. 79: 672-676.

Crump, J.A., P.M. Griffin, and F.J. Angulo. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. *Clin. Inf. Dis*. 35:859-865.

Darling Ingredients. 2016. Restaurants and supermarkets. Available at: https://www.darlingii.com/solutions/restaurant-supermarket/. Accessed 12 June 2016.

Dar Pro Solutions. N.d. Used cooking oil. Available at: https://d118ospkkl5uqf.cloudfront.net/DAR_PRO_Ingredients/DAR_PRO_I_Files/Ind%200614%20UsedCookingOil.pdf. Accessed 1 May 2016.

Department of Health and Human Services, Food and Drug Administration (FDA). 2012. Inspection observations made during inspection of Diamond Pet Food Processors of SC, LLC. Available at: http://www.fda.gov/downloads/AboutFDA/CentersOffices/OfficeofGlobalRegulatoryOperationsandPolicy/ORA/ORAElectronicReadingRoom/UCM304252.pdf. Accessed 18 May 2016.

Doyle, M.P. 1991. *Escherichia coli* O157:H7 and its significance in foods. *Int. J. Food. Microbiol*. 12:289-302.

Eirmann, L., C. Cowell, and L. Thompson. 2012. Pet food safety: The roles of government, manufacturers, and veterinarians. *Compendium* 34.1: E1-E3.

Finley, R., R. Reid-Smith, and J.S. Weese. 2006. Human health implications of *Salmonella*-contaminated natural pet treats and raw pet food. *Clin. Inf. Dis*. 42:686-691.

Finn, S., O. Condell, P. McClure, A. Amezquita, and S. Fanning. 2013. Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments. *Front. Microbiol*. 4:1-15.

Folch, J., M. Lees, and G.H.S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. 226:497-509.

Food and Agriculture Organization of the United Nations (FAO). 2009. How to feed the world in 2050. Available at: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf . Accessed 2 February 2016.

Food and Drug Administration (FDA). 2011. Food Safety Modernization Act. Pub. L. 124 Stat. 3885-3973. *GPO*. Available at: https://www.gpo.gov/fdsys/pkg/PLAW-111publ353/pdf/PLAW-111publ353.pdf. Accessed 2 May 2016.

Food and Drug Administration (FDA). 2012. Bad Bug Book. Available at: http://www.fda.gov/downloads/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/UCM297627.pdf. Accessed 4 May 2016.

Food and Drug Administration (FDA). 2013a. FDA issues compliance policy guide for *Salmonella* in food for animals. Available at: http://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm360834.htm. Accessed 18 May 2016.

Food and Drug Administration (FDA). 2013b. Compliance policy guide sec. 690.800 *Salmonella* in food for animals. Available at: http://www.fda.gov/downloads/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/UCM361105.pdf. Accessed 18 May 2016.

Food and Drug Administration (FDA). 2015. Current good manufacturing practice, hazard analysis, and risk-based preventive controls for food for animals. Available at: https://www.regulations.gov/#!documentDetail;D=FDA-2011-N-0922-0489. Accessed 15 June 2016.

Food and Drug Administration. 2016a. Frequently asked questions on FSMA. Available at: http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm247559.htm. Accessed 2 May 2016.

Food and Drug Administration. 2016b. FSMA final rule for preventive controls for animal food. Available at: http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm366510.htm. Accessed 30 April 2016.

Forsythe, S.J. 2010. The microbiology of safe food. Wiley-Blackwell, Hoboken, NJ.

Franco, D.A. 2002. Animal disposal. Available at http://www.rendermagazine.com/industry/animal-disposal/. Accessed 25 January 2016.

He, Y., D. Guo, J. Yang, M.L. Tortorello, and W. Zhang. 2011. Survival and heat resistance of *Salmonella enterica* and *Escherichia coli* O157:H7 in peanut butter. *Appl. Environ. Microb.* 77: 8434-8438.

Humphrey, T. 2004. *Salmonella*, stress responses and food safety. *Nature*. 2:504-509.

Interagency Food Safety Analytics Collaboration Project (IFSAC). 2015. Foodborne illness source attribution estimates for *Salmonella*, *Escherichia coli* O157 (*E. coli* O157), *Listeria*

monocytogenes (*Lm*) and *Campylobacter* using outbreak surveillance data. Available at: https://www.cdc.gov/foodsafety/pdfs/ifsac-project-report-508c.pdf. Accessed 19 July 2016.

Jekanowski, M. 2011. Survey says: A snapshot of rendering. Render Magazine. April 2011: 58-61.

Juneja, V.K., B.S. Eblen, and H.M. Marks. 2001. Modeling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. *Int. J. Food. Microbiol.* 70:37-51.

King, H. and G. Ades. 2015. Hazard analysis and risk-based preventive controls (HARPC): The new GMP for food manufacturing. Available at: http://www.foodsafetymagazine.com/magazine-archive1/octobernovember-2015/hazard-analysis-and-risk-based-preventive-controls-harpc-the-new-gmp-for-food-manufacturing/. Accessed 19 July 2016.

Laboratoires Expanscience. No date. What is an unsaponifiable? Accessed 4 May 2016. Available at: http://www.original-asu.com/what-is-an-unsaponifiable.html.

Leyer, G.J., L. Wang, and E.A. Johnson. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Appl. Environ. Microb.* 61:3752-3755.

Li, C., L. Huang, and J. Chen. 2014 Comparative study of thermal inactivation kinetics of *Salmonella* spp. in peanut butter and peanut butter spread. *Food Control*. 45:143-149.

Lim, J.Y., J.W. Yoon, and C.J. Hovde. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *J. Microbiol. Biotechnol.* 20:5-14.

Ma, L., G. Zhang, P. Gerner-Smidt, V. Mantripragada, I. Ezeoke, and M. P. Doyle. 2009. Thermal inactivation of *Salmonella* in peanut butter. *J. Food. Protect*. 72: 1596-1601.

Maberry, Tiffany. 2016. FSM scoop: Pet food safety. Available at: http://www.foodsafetymagazine.com/enewsletter/fsm-scoop-pet-food-safety/. Accessed 18 July 2016,

McGlashan, S. 2006. Industrial and energy uses of animal by-products, past and future. *In* D. L. Meeker (ed.), Essential rendering. National Renderers Association, Alexandria, VA. p. 1–16.

Mead, P. S. and P. M. Griffin. 1998. *Escherichia coli* O157:H7. *Lancet.* 352:1207-1212.

Meeker, D. L., and C. R. Hamilton. 2006. An overview of the rendering industry. *In* D. L. Meeker (ed.), Essential rendering. National Renderers Association, Alexandria, VA. p. 1–16.

Meeker, D. L., and J. L. Meisinger. 2015. Companion animal symposium: Rendered ingredients significantly influence sustainability, quality, and safety of pet food. *J. Anim. Sci*. 93: 835-847.

Murphy, R.Y., T. Osaili, L.K. Duncan, and J.A. Marcy. 2004. Thermal inactivation of *Salmonella* and *Listeria monocytogenes* in ground chicken thigh/leg meat and skin. *Poult Sci*. 83:1218-1225.

Nakano, M., E. Yamasaki, A. Ichoinose, T. Shimohata, J.K. Akada, K. Nakamura, J. Moss, T. Hirayama, and H. Kurazono. 2012. *Salmonella* enterotoxin (Stn) regulates membrane composition and integrity. *Dis. Model. Mech.* 5: 515-521.

National Renderers Association. 2015. Rendering is recycling. Available at: https://d10k7k7mywg42z.cloudfront.net/assets/53e623d1f002ff3e7c00027c/infographic_brochure_web.pdf. Accessed 11 April 2015.

Nelles, L.P., M.K. Sucan, and N.B. Trivedi. February 2008. Treatment of vegetable oils or animal fasts with sulfur or nitrogen donor compounds for animal food flavorings. U.S. patent 7,329,426.

Ng, H., H.G. Bayne, J.A. Garibaldi. 1969. Heat resistance of *Salmonella*: the uniqueness of *Salmonella* senftenberg 775W. *Appl. Microbiol.* 17:78-82.

Ochman, H. F.C. Soncini, F. Solomon, and E.A. Groisman. 1996. Identification of pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl. Acad. Sci.* 93: 7800-7804.

Ockerman, H.W. and L. Basu. 2014. Inedible. Encyclopedia of meat science. 2nd edition. Academic press. Waltham, Massachusetts. .

Osaili, T.M., C.L. Green, E.M. Martin, B.L. Beard, A.E. Keener, and J.A. Marcy. Thermal . inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in breaded pork patties. *J. Food. Sci*. 72:M56-M61.

Pacific Alternative Energy Resource, LLC. N.d. Yellow grease, used cooking oil and waste vegetable oil. Available at: http://pacaltenergy.com/uco.html. Accessed 20 March 2016.

Plattner B. and G. Rokey. 2007. Controlling ingredient interactions; How raw materials impact extrusion processes and costs. Available at: http://www.petfoodindustry.com/articles/533-controlling-ingredient-interactions. Accessed 20 March 2016.

Revox Sterilization Solutions. 2014. Determining D-values: The importance of standardized measurement. Available at: http://www.revoxsterilization.com/article/determining-d-value-the-importance-of-importance-standardized-measurements. Accessed 30 May 2016.

Ryu, J. and L.R. Beuchat. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int. J. Food. Microbiol.* 45:185-193.

Scallan, E. R.M. Hoeskstra, F.J. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin. 2011. Foodborne illness acquired in the United States—Major pathogens. *Emerg. Infect. Dis*. 17: 7-15.

Schultz, M. 2008. Theobald Smith. *Emerg. Infect. Dis.* 14: 1940-1942.

Silliker. 2014. FSMA: A new regulatory paradigm for pet food safety. Available at: http://cdn2.hubspot.net/hub/240373/file-629184820-pdf/Landing_Pages/Documents/FSMA-_A_New_Regulatoy_Paradigm_for_the_Pet_Food_Safety.pdf?t=1396296701000. Accessed 10 May 2016.

Stopforth, J.D., R. Suhalim, B. Kottapalli, W.E. Hill, and M. Samadpour. 2008. Thermal inactivation D- and z-values of multidrug-resistant and non-multidrug-resistant *Salmonella* serotypes and survival in ground beef exposed to consumer-style cooking. *J. Food. Protect*. 71:509-515.

Strauss, D.M. 2011. An analysis of the FDA Food Safety Modernization Act: Protection for consumers and boon for business. *Food Drug Law J*. 66: 353-376.

Stringer, S.C., S.M. George, and M.W. Peck. 2000. Thermal inactivation of *Escherichia coli* O157:H7. *J. Appl. Microbiol.* 88: 79S-89S.

Swisher, K. 2013.US rendering: A $10 billion industry. Render magazine. April 2013: 10-17.

Tarr, P.I., C.A. Gordon, W.L Chandler. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet*. 365:1073-1086.

Todar, T. 2008. Pathogenic *E. coli.* Available at: http://textbookofbacteriology.net/e.coli.html. Accessed 28 May 2016.

US General Accounting Office. 2000. Food safety: controls can be strengthened to reduce the risk of disease linked to unsafe animal feed. Washington, DC: US General Accounting Office. Report GAO/RCED-00-255.